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Detection of Amphotericin B Resistance in *Candida haemulonii* and Closely Related Species by Use of the Etest, Vitek-2 Yeast Susceptibility System, and CLSI and EUCAST Broth Microdilution Methods

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The emerging fungal pathogens *Candida haemulonii* and *Candida pseudohaemulonii* often show high-level resistance to amphotericin B (AMB). We compared the utilities of five antifungal susceptibility testing methods, i.e., the Etest using Mueller-Hinton agar supplemented with glucose and methylene blue (Etest-MH), the Etest using RPMI agar supplemented with glucose (Etest-RPG), the Vitek-2 yeast susceptibility system, and the Clinical and Laboratory Standards Institute (CLSI) and European Committee on Antimicrobial Susceptibility Testing (EUCAST) broth microdilution methods, for the detection of AMB-resistant isolates of *C. haemulonii* and closely related species. Thirty-eight clinical isolates (8 *C. haemulonii*, 10 *C. pseudohaemulonii*, and 20 *Candida auris* isolates) were analyzed. Of the 18 *C. haemulonii* and *C. pseudohaemulonii* isolates, 18, 15, 18, 10, and 9 exhibited AMB MICs of >1 μg/ml by the Etest-MH, Etest-RPG, Vitek-2, CLSI, and EUCAST methods, respectively. All 20 *C. auris* isolates showed AMB MICs of ≤1 μg/ml by all five methods. Of the methods, the Etest-MH generated the broadest distribution of AMB MICs for all 38 isolates and showed the best discrimination between the *C. haemulonii* and *C. pseudohaemulonii* isolates (4 to 32 μg/ml) and those of *C. auris* (0.125 to 0.5 μg/ml). Taking the Etest-MH as the reference method, the essential agreements (within two dilutions) for the Etest-RPG, Vitek-2, CLSI, and EUCAST methods were 84, 92, 55, and 55%, respectively; the categorical agreements were 92, 92, 79, and 76%, respectively. This study provides the first data on the efficacy of the Etest-MH and its excellent agreement with Vitek-2 for discriminating AMB-resistant from AMB-susceptible isolates of these *Candida* species.

Although it has been used for many years, few clinical *Candida* isolates have been reported to be resistant to amphotericin B (AMB). However, we recently described the emergence of *Candida haemulonii* and closely related species at several hospitals in Korea (8, 12). Although sequencing confirmed that the isolates were *C. haemulonii* group I, *Candida pseudohaemulonii*, or *Candida auris*, all were identified as *C. haemulonii* by the Vitek-2 YST yeast card system (bioMérieux, Marcy l'Étoile, France), and they were genetically closely related compared to other yeasts in the phylogenetic tree (12). Fungemia was the most common clinical presentation (8, 10). Some blood isolates of *C. haemulonii* and *C. pseudohaemulonii* from fungemic patients exhibited high-level *in vitro* resistance to AMB and were associated with AMB therapeutic failure (8). These cases demonstrate that the detection of AMB resistance is essential for the treatment of severe infections caused by AMB-resistant *Candida* species.

The limitations of the Clinical and Laboratory Standards Institute (CLSI) reference methodology for detecting AMB resistance are well documented, and several alternative methods, including the Etest, the European Committee on Antimicrobial Susceptibility Testing (EUCAST) method, and the Vitek-2 yeast susceptibility system (bioMérieux), have been proposed and evaluated (1–3, 5, 6, 17). However, the evaluation of AMB susceptibility testing methods for *Candida* species is not easy, because only a few clinical isolates of *Candida* species have been reported to be resistant to AMB. Although the Etest has been shown to be superior to the CLSI M27-A broth dilution test for discriminating between AMB-susceptible and -resistant isolates of *Candida* species (9, 14, 15), studies that have compared AMB MICs among CLSI and Etest, EUCAST, or Vitek-2 have rarely reported major discrepancies, probably owing to the small number of true AMB-resistant isolates in their collections (1, 5, 6, 17).

In this study, by testing clinical isolates of *C. haemulonii* and closely related species, we evaluated the ability of five antifungal susceptibility testing methods, i.e., the Etest on Mueller-Hinton agar supplemented with glucose and methylene blue (Etest-MH), the Etest on RPMI agar supplemented with glucose (Etest-RPG), the Vitek-2 yeast susceptibility test, and the CLSI and EUCAST broth microdilution methods, for the detection of AMB resistance in *Candida* isolates. Our study provides the first data on the ability of the Etest-MH to provide the best discrimination between AMB-resistant and -susceptible isolates of these fungi. In addition, the Vitek-2 system showed excellent agreement with the Etest-MH, suggesting that this system is a useful tool for detecting AMB resistance in *Candida* isolates.

Received 17 November 2011 **Returned for modification** 7 January 2012
Accepted 13 March 2012
Published ahead of print 21 March 2011
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doi:10.1128/JCM.06440-11

TABLE 1 Distribution of amphotericin B MICs for 38 clinical isolates of *Candida haemulonii* and closely related species as determined by five methods

Species (no. of isolates)	Test system[a]	No. of isolates for which amphotericin B MIC (μg/ml) was[b]:														
		0.125	0.19	0.25	0.38	0.5	0.75	1	1.5	2	4	6	8	12	16	32
C. haemulonii (8)	Etest-MH										1			1	1	5
	Etest-RPG					1			2	1	1			1		2
	Vitek-2									1	2		5			
	CLSI							6		2						
	EUCAST							7		1						
C. pseudohaemulonii (10)	Etest-MH											1		1		8
	Etest-RPG					1		1								8
	Vitek-2									2			3		5	
	CLSI							2		1	7					
	EUCAST							2		6	2					
C. auris (20)	Etest-MH	1	1	7	5	6										
	Etest-RPG			5	6	5	2	2								
	Vitek-2			13		5		2								
	CLSI			2		13		5								
	EUCAST			2		15		3								
Total (38)	Etest-MH	1	1	7	5	6					1	1		2	1	13
	Etest-RPG			5	6	7	2	3	2	1	1			1		10
	Vitek-2			13		5		2		3	2		8		5	
	CLSI			2		13		13		3	7					
	EUCAST			2		15		12		7	2					

[a] Etest-MH, Etest on Mueller-Hinton agar supplemented with glucose and methylene blue; Etest-RPG, Etest on RPMI agar supplemented with glucose; CLSI, CLSI M27-A3 broth microdilution method; EUCAST, European Committee on Antimicrobial Susceptibility Testing broth microdilution method.

[b] Each isolate was tested at least twice; the AMB MIC results for the final runs of each isolate are shown. Isolates with MICs of 32 μg/ml by both the Etest-MH and Etest-RGP methods encompass all isolates for which MICs were 32 μg/ml or more, while isolates with MICs of 16 μg/ml by the Vitek-2 system include all isolates for which MICs were 16 μg/ml or more.

MATERIALS AND METHODS

***Candida* isolates.** Thirty-eight clinical isolates of *C. haemulonii* and closely related species, i.e., *C. haemulonii* group I ($n = 8$), *C. pseudohaemulonii* ($n = 10$), and *C. auris* ($n = 20$), were tested. The isolates were recovered from 38 patients at seven Korean university hospitals between 1996 and 2010. The *C. haemulonii* group I isolates were cultured from blood ($n = 6$), respiratory specimens ($n = 1$), and a central venous catheter ($n = 1$); *C. pseudohaemulonii* isolates were from blood ($n = 9$) or urine ($n = 1$) cultures; and *C. auris* isolates were from ear specimens ($n = 17$) and blood cultures ($n = 3$). All isolates were identified by sequencing the internal transcribed spacer (ITS) and D1/D2 regions of the 26S ribosomal DNA (8).

Antifungal susceptibility testing procedures. AMB MICs for each isolate were determined by five antifungal susceptibility testing methods. The Etest (bioMérieux) was performed on the following two media, as described previously (16): (i) Mueller-Hinton agar supplemented with glucose (2%) and methylene blue (0.5 μg/ml) (Etest-MH) and (ii) RPMI agar supplemented with 2% glucose (Etest-RPG). The Etest AMB MICs on both media were read after 48 h of incubation at 35°C and were determined to be at 100% inhibition of growth where the border of the elliptical inhibition zone intersected the scale of the strip edge. For Vitek-2 yeast susceptibility tests (AST-YS01 Vitek 2 cards), susceptibility testing, reading, and interpretations of the results were performed in accordance with the manufacturer's instructions. AMB MICs were determined by the CLSI M27-A3 broth microdilution after 48 h of incubation, which was defined as complete inhibition of growth (4). For testing by the EUCAST method, the AMB MIC endpoints were determined after 24 h of incubation and defined as the lowest drug concentration that resulted in a reduction in growth of ≥90% compared to that in a drug-free control well (19). Each isolate was tested twice, and testing was repeated in cases of two MIC results that were not within two dilutions. The results of repeat runs of each isolate were accepted as final. Two reference strains, *Candida parapsilosis* ATCC 22019 (AMB MICs, 0.25 to 1 μg/ml) and *Candida krusei* ATCC 6258 (AMB MICs, 0.25 to 2 μg/ml), were used as quality-control isolates for each antifungal susceptibility test. In addition, *C. pseudohaemulonii* CBS 12370 (KCTC 17807), which exhibited AMB MICs of ≥2 μg/ml by all five methods, was used as a control strain in each test.

Analysis of results. No AMB susceptibility breakpoint has been established for these *Candida* species, but ≤1 μg/ml has been suggested (11, 13, 16). On this basis, we adopted putative AMB breakpoints for both CLSI and EUCAST (susceptible, ≤1 μg/ml; resistant, ≥2 μg/ml) and the two Etests (susceptible, ≤1 μg/ml; resistant, ≥1.5 μg/ml). In addition, we used the AMB breakpoints provided by the Vitek-2 system (susceptible, ≤1 μg/ml; intermediate, 2 μg/ml; resistant, ≥4 μg/ml). The Etest MICs were rounded to the next higher CLSI or EUCAST concentration to simplify comparison, and discrepancies among MIC endpoints of ≥2 dilutions (two wells) were used to calculate the essential agreement. Categorical agreement was defined as the percentage of isolates classified into the same category by both methods. Discrepancies were considered to be very major if an isolate classified as resistant by the reference method was categorized as susceptible by the other method, major if an isolate classified as susceptible by the reference method was classified as resistant by the other method, and minor if an isolate was classified as susceptible or resistant by one method and intermediate by the other. Statistical analyses were performed using SPSS for Windows version 18.0. P values of <0.05 were considered significant.

RESULTS AND DISCUSSION

Although rarely isolated in clinical microbiology laboratories, *C. haemulonii* and *C. pseudohaemulonii* have consistently been reported to be resistant to AMB (7, 8, 18, 20). We evaluated the ability of five antifungal susceptibility testing methods to detect AMB resistance *in vitro* in clinical isolates of *C. haemulonii* and

TABLE 2 Agreement between amphotericin B results obtained by the Etest-MH and each of the other methods for 38 isolates of *Candida haemulonii* and closely related species

Species (no. of isolates)	Test system	% Essential agreement[a]	No. of isolates with result[b]			% Categorical agreement with Etest-MH	% of category errors		
			Susceptible	Intermediate	Resistant		Very major	Major	Minor
C. haemulonii (8)	Etest-MH		0	0	8				
	Etest-RPG	75	1	0	7	88	13		
	Vitek-2	75	0	1	7	88	0		13
	CLSI	0	6	0	2	26	75		
	EUCAST	0	7	0	1	13	88		
C. pseudohaemulonii (10)	Etest-MH		0	0	10				
	Etest-RPG	80	2	0	8	80	20		
	Vitek-2	90	0	2	8	80	0		20
	CLSI	0	2	0	8	80	20		
	EUCAST	0	2	0	8	80	20		
C. auris (20)	Etest-MH		20	0	0				
	Etest-RPG	100	20	0	0	100	0		
	Vitek-2	100	20	0	0	100	0		
	CLSI	100	20	0	0	100	0		
	EUCAST	100	20	0	0	100	0		
Total (38)	Etest-MH		20	0	18				
	Etest-RPG	84	23	0	15	92	8		
	Vitek-2	92	20	3	15	92	0		8
	CLSI	55	28	0	10	79	21		
	EUCAST	55	29	0	9	76	24		

[a] Essential agreement (± 2 $\log_2$ dilutions) between Etest-MH and each of other four methods.
[b] Putative AMB breakpoints were used for two Etests (susceptible, ≤ 1 μg/ml; resistant, ≥ 1.5 μg/ml) and for both the EUCAST and CLSI methods (susceptible, ≤ 1 μg/ml; resistant, ≥ 2 μg/ml), but AMB breakpoints provided by the Vitek-2 system (susceptible, ≤ 1 μg/ml; intermediate, 2 μg/ml; resistant, ≥ 4 μg/ml) were used for the Vitek-2 assay.

C. pseudohaemulonii in comparison with *C. auris*, which is an unusual *Candida* species most closely related to *C. haemulonii*. Table 1 shows the distribution of AMB MICs for 38 isolates of *C. haemulonii* and closely related species determined by the five methods. All 20 *C. auris* isolates showed AMB MICs of ≤ 1 μg/ml by all methods. However, of 18 isolates of *C. haemulonii* and *C. pseudohaemulonii*, 18 (100%), 15 (83%), 18 (100%), 10 (56%), and 9 (50%) had AMB MICs of >1 μg/ml by the Etest-MH, Etest-RPG, Vitek-2, CLSI, and EUCAST, respectively. These data suggest that *C. haemulonii* and *C. pseudohaemulonii* are innately resistant to AMB and that the Etest-MH and the Vitek-2 system have the ability to detect AMB resistance in these fungi.

Previously, we described seven cases of *C. pseudohaemulonii* fungemia and one of *C. haemulonii* fungemia at two hospitals in Korea (8). Blood isolates from these eight patients showed variable AMB susceptibility patterns (MIC = 0.5 to 32 μg/ml), as determined by the Etest-RPG. All of these isolates were included in the present study. Of the eight patients, three suffered AMB therapeutic failure and ultimately died. Blood isolates from two patients had AMB MICs of 32 μg/ml. Notably, one patient who had severe immunodeficiency died despite 4 days of AMB therapy, but the isolate showed an AMB MIC of 0.5 μg/ml. In the present study, this isolate was categorized as AMB susceptible (MIC = 0.5 μg/ml) by the Etest-RPG but as AMB resistant (MIC = 12 μg/ml) by the Etest-MH, illustrating the ability of the Etest-MH to detect AMB-resistant isolates and thereby to predict therapeutic resistance to AMB treatment. Overall, the AMB MICs of all 18 *C. haemulonii* and C. *pseudohaemulonii* isolates generated by the Etest-RPG showed susceptibility patterns of 0.5 to 32 μg/ml, similar to those in our previous report (8), while those generated by the Etest-MH ranged from 4 to 32 μg/ml, all of which were categorized as AMB resistant. These results indicate that Mueller-Hinton agar supplemented with glucose and methylene blue may better support the growth of *C. haemulonii* and *C. pseudohaemulonii* than RPMI agar supplemented with glucose and thereby provide superior detection of AMB-resistant isolates of *C. haemulonii* and *C. pseudohaemulonii*.

The CLSI M27 method is known to yield a narrow range of AMB MICs and therefore may be unable to discriminate resistant from susceptible *Candida* isolates (11). In our study, the CLSI and EUCAST methods generated similar ranges of AMB MICs (0.25 to 4 μg/ml), which were the narrowest of the five methods (Table 1). The Etest-MH generated the broadest distribution of AMB MICs, ranging from 0.125 to 32 μg/ml, followed by the Etest-RPG (0.25 to 32 μg/ml) and Vitek-2 (0.25 to 16 μg/ml). The AMB MICs determined by the Etest-MH easily distinguished all 18 isolates of *C. haemulonii* and *C. pseudohaemulonii* (4 to 32 μg/ml) from all 20 *C. auris* isolates (0.125 to 0.5 μg/ml). The AMB MICs determined by the Vitek-2 also distinguished all 18 isolates of *C. haemulonii* and *C. pseudohaemuloni* (2 to 16 μg/ml) from all 20 *C. auris* isolates (0.25 to 1 μg/ml). However, the AMB MICs for the *C. haemulonii* and *C. pseudohaemulonii* isolates determined by the other three methods (Etest-RPG, EUCAST, and CLSI) overlapped with those of *C. auris*. These data suggest that the Etest-MH provides the best discrimination between AMB-resistant and -susceptible isolates, followed by the Vitek-2 system.

When the Etest-MH was used as the reference standard, there were essential agreements of 55% with both the CLSI and EUCAST methods, which were significantly lower than those with

the Etest-RPG (84%) or Vitek-2 (92%) ($P < 0.05$). The categorical agreements for the Etest-RPG, Vitek-2, CLSI, and EUCAST methods were 92, 92, 79, and 76%, respectively (Table 2). The percentages of very major errors were 21% (8/38) between the Etest-MH and CLSI methods and 24% (9/38) between the Etest-MH and EUCAST methods. Vitek-2 produced no very major or major errors and only three (8%) minor errors. These three minor errors occurred as a result of isolates that were categorized as resistant by the Etest-MH but intermediate (MIC = 2 μg/ml) by Vitek-2. These findings suggest that the Vitek-2 with an adjusted AMB MIC breakpoint (resistant, ≥2 μg/ml) is more sensitive for detecting emerging resistance among these *Candida* species. Overall, these findings indicate that both the Etest and Vitek-2 are superior to the CLSI or EUCAST broth microdilution method for detecting AMB-resistant *Candida* isolates. Vitek-2 antifungal susceptibility testing is a fully automated commercial method that can rapidly determine AMC MICs. Therefore, our study suggests that Vitek-2 is a useful screening method for detecting AMB resistance in *Candida* isolates.

ACKNOWLEDGMENT

This study was supported by the Basic Science Research Program through the National Research Foundation of Korea (NRF) funded by the Ministry of Education, Science and Technology (2010-0021556).

REFERENCES

1. **Alexander B, et al.** 2007. Comparative evaluation of Etest and Sensititre YeastOne panels against the Clinical and Laboratory Standards Institute M27-A2 reference broth microdilution method for testing *Candida* susceptibility to seven antifungal agents. J. Clin. Microbiol. **45**:698–706.
2. **Arikan S.** 2007. Current status of antifungal susceptibility testing methods. Med. Mycol. **45**:569–587.
3. **Bourgeois N, et al.** 2010. Antifungal susceptibility of 205 *Candida* spp. isolated primarily during invasive candidiasis and comparison of the Vitek 2 system with the CLSI broth microdilution and Etest methods. J. Clin. Microbiol. **48**:154–161.
4. **Clinical and Laboratory Standards Institute.** 2008. Reference method for broth dilution antifungal susceptibility testing of yeasts; approved standard M27-A3, 3rd ed. Clinical and Laboratory Standards Institute, Wayne, PA.
5. **Cuenca-Estrella M, et al.** 2002. Comparative evaluation of NCCLS M27-A and EUCAST broth microdilution procedures for antifungal susceptibility testing of *Candida* species. Antimicrob. Agents Chemother. **46**:3644–3647.
6. **Cuenca-Estrella M, et al.** 2010. Comparison of the Vitek 2 antifungal susceptibility system with the clinical and laboratory standards institute (CLSI) and European Committee on Antimicrobial Susceptibility Testing (EUCAST) broth microdilution reference methods and with the Sensititre YeastOne and Etest techniques for in vitro detection of antifungal resistance in yeast isolates. J. Clin. Microbiol. **48**:1782–1786.
7. **Khan ZU, et al.** 2007. Outbreak of fungemia among neonates caused by *Candida haemulonii* resistant to amphotericin B, itraconazole, and fluconazole. J. Clin. Microbiol. **45**:2025–2027.
8. **Kim MN, et al.** 2009. *Candida haemulonii* and closely related species at 5 university hospitals in Korea: identification, antifungal susceptibility, and clinical features. Clin. Infect. Dis. **48**:e57–61.
9. **Krogh-Madsen M, Arendrup MC, Heslet L, Knudsen JD.** 2006. Amphotericin B and caspofungin resistance in *Candida glabrata* isolates recovered from a critically ill patient. Clin. Infect. Dis. **42**:938–944.
10. **Lee WG, et al.** 2011. The first three reported cases of nosocomial fungemia caused by *Candida auris.* J. Clin. Microbiol. **49**:3139–3142.
11. **Nguyen MH, et al.** 1998. Do in vitro susceptibility data predict the microbiologic response to amphotericin B? Results of prospective study of patients with *Candida* fungemia. J. Infect. Dis. **177**:425–430.
12. **Oh BJ, et al.** 2011. Biofilm formation and genotyping of *Candida haemulonii*, *Candida pseudohaemulonii*, and a proposed new species (*Candida auris*) isolates from Korea. Med. Mycol. **49**:98–102.
13. **Park BJ, et al.** 2006. Evaluation of amphotericin B interpretive breakpoints for *Candida* bloodstream isolates by correlation with therapeutic outcome. Antimicrob. Agents Chemother. **50**:1287–1292.
14. **Peyron F, et al.** 2001. Improved detection of amphotericin B-resistant isolates of *Candida lusitaniae* by Etest. J. Clin. Microbiol. **39**:339–342.
15. **Pfaller MA, Messer SA, Bolmström A.** 1998. Evaluation of Etest for determining in vitro susceptibility of yeast isolates to amphotericin B. Diagn. Microbiol. Infect. Dis. **32**:223–227.
16. **Pfaller MA, et al.** 2004. Evaluation of the Etest method using Mueller-Hinton agar with glucose and methylene blue for determining amphotericin B MICs for 4,936 clinical isolates of *Candida* species. J. Clin. Microbiol. **42**:4977–4979.
17. **Pfaller MA, Diekema DJ, Procop GW, Rinaldi MG.** 2007. Multicenter comparison of the VITEK 2 antifungal susceptibility test with the CLSI broth microdilution reference method for testing amphotericin B, flucytosine, and voriconazole against *Candida* spp. J. Clin. Microbiol. **45**:3522–3528.
18. **Rodero L, et al.** 2002. Transient fungemia caused by an amphotericin B-resistant isolate of *Candida haemulonii.* J. Clin. Microbiol. **40**:2266–2269.
19. **Rodriguez-Tudela JL, et al.** 2008. EUCAST definitive document EDef 7.1: method for the determination of broth dilution MICs of antifungal agents for fermentative yeasts. Clin. Microbiol. Infect. **14**:398–405.
20. **Sugita T, Takashima M, Poonwan N, Mekha N.** 2006. *Candida pseudohaemulonii* sp. nov., an amphotericin B- and azole-resistant yeast species, isolated from the blood of a patient from Thailand. Microbiol. Immunol. **50**:469–473.